EXPERT REVIEWS

Monovalent virus-like particle vaccine protects guinea pigs and nonhuman primates against infection with multiple Marburg viruses

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Background: Virus-like particle (VLP)-based vaccines have the advantage of being morphologically and antigenically similar to the live virus from which they are derived. Expression of the glycoprotein and VP40 matrix protein from *Lake Victoria marburgvirus* (MARV) results in spontaneous production of VLPs in mammalian cells. Guinea pigs vaccinated with Marburg virus VLPs (mVLPs) or inactivated MARV (iMARV) develop homologous humoral and T-cell responses and are completely protected from a lethal homologous MARV challenge. **Aims & methods:** To determine whether mVLPs based on the Musoke (aka Lake Victoria) isolate of MARV could broadly protect against diverse isolates of MARV, guinea pigs were vaccinated with mVLPs or iMARV-Musoke and challenged with MARV-Musoke, -Ravn or -Ci67. **Results:** Prior to challenge, the mVLP- and iMARV-vaccinated guinea pigs had high levels of homologous MARV-Musoke and heterologous MARV-Ravn and -Ci67 antibodies. The Musoke-based mVLPs and iMARV vaccines provided complete protection in guinea pigs against viremia, viral replication and pathological changes in tissues, and lethal disease following challenge with MARV-Musoke, -Ravn or -Ci67. Guinea pigs vaccinated with RIBI adjuvant alone and infected with guinea pig-adapted MARV-Musoke, -Ravn or -Ci67 had histopathologic findings similar to those seen in the nonhuman primate model for MARV infection. Based on the strong protection observed in guinea pigs, we next vaccinated cynomolgus macaques with Musoke-based mVLPs and showed the VLP-vaccinated monkeys were broadly protected against three isolates of MARV (Musoke, Ravn and Ci67). **Conclusion:** Musoke mVLPs are effective at inducing broad heterologous immunity and protection against multiple MARV isolates.

KEYWORDS: antibody • Ebola • filovirus • Marburg • nonhuman primate • protective immunity • vaccine • virus-like particle

Marburg virus (MARV) is a member of the *Filoviridae* family and causes severe viral hemorrhagic disease with high mortality rates [1]. Recently, several efficacious candidate vaccines for the filoviruses have been generated. Classical methods of vaccine production include attenuated and inactivated viral preparations. However, these approaches would be too costly due to biosafety level (BSL)-4 production requirements and also dangerous due to the potential for viral revertants or incomplete viral inactivation [2–5]. Several vectored vaccine approaches for MARV have been successful in protecting nonhuman primates (NHPs) from lethal disease. The first demonstration of complete protection against a filovirus infection in NHPs was based on the Venezuelan equine encephalitis replicon particle, where the antigen of interest, in this case the MARV glycoprotein (GP), is inserted in place of the structural genes [6]. A recombinant vesicular stomatitis virus vaccine encoding MARV GP protects NHPs against multiple isolates of MARV [7,8]. A replication-deficient adenovirus-based vaccine

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14. ABSTRACT

Virus-like particle (VLP)-based vaccines have the advantage of being morphologically and antigenically similar to the live virus from which they are derived. Expression of the glycoprotein and VP40 matrix protein from Lake Victoria marburgvirus (MARV) results in spontaneous production of VLPs in mammalian cells. Guinea pigs vaccinated with marburgvirus (m)VLPs or inactivated MARV (iMARV) develop homologous humoral and T cell responses and are completely protected from a lethal homologous MARV challenge. To determine whether the mVLPs, based on the Musoke (aka Lakevictoria) isolate of MARV, could broadly protect against diverse isolates of MARV, guinea pigs were vaccinated with mVLPs or inactivated MARV-Musoke and challenged with MARV-Musoke, -Ravn, or Ci67. Prior to challenge, the mVLP- and iMARV-vaccinated guinea pigs had high levels of homologous MARV-Musoke and heterologous MARV-Ravn and Ci67 antibodies. The Musoke-based mVLPs and iMARV vaccines provided complete protection in guinea pigs against viremia, viral replication and pathological changes in tissues, and lethal disease following challenge with MARV-Musoke, -Ravn, or Ci67. Guinea pigs vaccinated with RIBI adjuvant alone and infected with guinea pig-adapted MARV- Musoke, -Ravn, or Ci67 had histopathologic findings similar to those seen in the nonhuman (NHP) model for MARV virus infection. Based on the strong protection observed in guinea pigs, we next vaccinated cynomolgus macaques with Musoke-based mVLPs and showed the VLP-vaccinated monkeys were broadly protected against three isolates of MARV (Musoke, Ravn, and Ci67). Thus, Musoke marburgvirus-based VLPs are effective at inducing broad heterologous immunity and protection against multiple MARV isolates.

15. SUBJECT TERMS
filovirus, Marburg, virus-like particles, vaccine, monovalent, efficacy, laboratory animals, guinea pigs, nonhuman primates

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can protect guinea pigs and NHPs against Ebola virus (EBOV) and MARV [9,10]. Each of these candidate vaccines presents with concerns, for example, acceptable vaccine doses, vaccine safety and the impact of prior immunity to the vaccine vector.

While the majority of work developing filovirus vaccines has utilized virus vectors, several studies demonstrate that subunit vaccines can safely and specifically protect against MARV [11,12]. While vaccination of guinea pigs or NHPs with either a recombinant baculovirus-produced GP or DNA vaccine encoding GP alone is not entirely efficacious, a prime–boost strategy with both the DNA vaccine and baculovirus-produced recombinant GP successfully protected animals from both lethal EBOV and MARV infection [2,13,14]. Our laboratory has taken advantage of the phenomenon that MARV virus-like particles (mVLPs) are produced spontaneously in MARV GP- and matrix protein VP40-transfected mammalian cells, and we are working to develop a mVLP-based vaccine [12,15–18].

Guinea pigs vaccinated with mVLPs (based on the Musoke isolate) in RIBI adjuvant generate high levels of serum virus-specific and -neutralizing antibodies, and proliferative recall responses against MARV *in vitro* requiring CD4+ T cells [18]. After infection with homologous MARV-Musoke, mVLP-vaccinated guinea pigs are protected completely from clinical symptoms, viremia and death [16,18]. Studies revealed that MARV GP, but not VP40, was required and sufficient to protect against MARV-Musoke challenge. VLPs comprised of MARV GP and the EBOV VP40 protected against MARV challenge, while VLPs containing EBOV GP and MARV VP40 did not protect against lethal disease [16].

To determine whether the Musoke-based mVLPs offer broad protection and function as a pan-MARV vaccine, we assessed immune responses and protection against MARV-Musoke, -Ravn or -Ci67 in Musoke mVLP-vaccinated guinea pigs. These MARV strains differ up to 22% at the amino acid level within the GP, which is the viral attachment protein and presumably the only protein required to vaccinate against for a protective immune response. The data presented here indicate that Musoke-based VLPs induce broad immunity against multiple MARV strains in guinea pigs and NHPs.

Methods
Virus & cells
MARV-Musoke, -Ravn and -Ci67 were propagated and enumerated by standard plaque assay on Vero E6 cells [19]. The adaptation to lethality of the MARV-Musoke, -Ravn and -Ci67/Popp strains took seven, two and two serial passages through guinea pigs, respectively [6,13,20]. MARV-infected cells and guinea pigs were handled under maximum containment in a BSL-4 laboratory at the US Army Medical Research Institute of Infectious Diseases (USAMRID; MD, USA).

Vaccine preparations
Marburg virus VLPs were prepared essentially as described previously [15,17,18]. Briefly, for the guinea pig studies, 293T cells were cotransfected with individual pWRG vectors encoding for MARV VP40 and GP using Lipofectamine™ 2000 (Invitrogen, CA, USA). Alternatively, for the NHP studies, baculovirus recombinants were used to generate insect cell-derived mVLPs, in a similar manner to our previous work [21]. After 48–72 h, cell supernatants from the 293T or insect cells were collected, cleared of cellular debris by low-speed centrifugation and then pelleted at $9500 \times g$ for 4 h. The crude VLP preparations were separated on a 20–60% continuous sucrose gradient, concentrated by a second centrifugation and resuspended in endotoxin-free phosphate-buffered saline (PBS). MARV-Musoke virus preparations were grown in Vero E6 cells and purified in a similar manner to the VLPs. The sucrose-purified virus preparation was inactivated by irradiation with 1×10^7 rads. Gradient fractions containing mVLPs or inactivated MARV (iMARV) were determined by western blotting and electron microscopy. Total protein concentrations of the vaccine preparations were determined in the presence of NP40 detergent using a detergent-compatible protein assay (BioRad, CA, USA). Endotoxin levels in all vaccine preparations used in this study were less than 0.03 endotoxin units, as determined by the Limulus amebocyte lysate test (Biowhittaker, MD, USA).

Guinea pig vaccinations
Inbred strain 13 guinea pigs (USAMRIID), aged 6–9 months and of both sexes, were randomized into groups, with each guinea pig identified using a radiotransponder microchip (Bio-Medic Data Systems, Inc., DE, USA) inserted underneath the skin. Guinea pigs were vaccinated intramuscularly with 50 µg of mVLPs or iMARV with 200 µl of RIBI containing monophosphoryl lipid, synthetic trehalose dicorynomycolate and cell wall skeleton emulsion (Corixa Corporation, MT, USA) diluted in endotoxin-free PBS on days 0, 21 and 42. Control guinea pigs were vaccinated with RIBI adjuvant in PBS alone. Serum samples were obtained from each guinea pig immediately before each vaccination (first [day 21], second [day 42] or third [day 63] vaccination) and 4 weeks after challenge (day 91). Research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adhered to principles stated in the Guide for the Care and Use of Laboratory Animals [22]. The facility where this research was conducted is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

Guinea pig viral challenge & postchallenge sampling
The guinea pigs were challenged subcutaneously 30 days after the third vaccination (day 72) with approximately 1000 plaque-forming units (pfu) of guinea pig-adapted MARV-Musoke, -Ravn or -Ci67 diluted in PBS. The guinea

pigs were bled on 7 days postchallenge from the retro-orbital sinus to obtain plasma for determination of circulating viral titers. After challenge, guinea pigs were observed at least twice daily for illness and death.

Guinea pig necropsy, histology & immunohistochemistry

Two animals randomly selected from each group were euthanized on 6–7 days postchallenge for macroscopic and microscopic evaluation. These guinea pigs were removed from the survival-analysis portion of the experiment. Complete necropsies were performed and the tissues from each guinea pig were collected in 10% neutral buffered formalin and held in the BSL-4 laboratory for over 30 days. The tissues were removed from the BSL-4 suite in fresh formalin, trimmed, embedded in paraffin, sectioned at 5–6 µm and placed on positive-charged glass slides. The sections were stained with hematoxylin and eosin for routine light microscopy or an immunoperoxidase assay (EnVision™, DAKO, CA, USA) using a 50:50 cocktail of mouse monoclonal antibodies against MARV NP. Briefly, the unstained sections were blocked with 0.6% hydrogen peroxide in methanol, pretreated with proteinase K for 6 min, and blocked a second time with a serum-free protein (DAKO) containing 5% normal goat serum for 30 min. The monoclonal cocktail was applied for 30 min at a dilution of 1:1200. The tissue was exposed to the EnVision peroxidase-labeled polymer for 30 min at room temperature, exposed to the substrate-chromagen DAB (DAKO), rinsed, counterstained with hematoxylin and coverslipped with Permount™.

Nonhuman primate studies

The cynomolgus macaques used in this study were found to be filovirus-, simian T-cell leukemia virus-1-, SIV- and herpes B antibody-negative in testing prior to initiation of the study. The VLP-vaccinated monkeys received three intramuscular injections at 42-day intervals containing 1 mg of VLPs and 0.1 ml of QS-21 adjuvant (kindly provided by Antigenics, MA, USA). Blood samples were obtained under anesthesia from the femoral vein of monkeys. Male and female cynomolgus macaques of approximately 3–4 kg in weight were challenged with approximately 1000 pfu of MARV-Musoke, -Ci67 or -Ravn via subcutaneous injection. Viremia was assayed by traditional plaque assay [19]. Hematological and kidney/liver-associated enzymes were measured as described previously [23,24].

Antibody titers

Levels of MARV-specific antibodies were determined as described previously [16,18]. Briefly, the wells were coated with sucrose-purified iMARV-Musoke, -Ravn or -Ci67 virions. End point titers were established as the inverse of the last dilution where the optical density of the sample was at least 0.2 greater than the corresponding control wells (irrelevant heterologous antigen). Convalescent serum samples removed from the BSL-4 laboratory were γ-irradiated with 2×10^6 rads from a ^{60}Co source before analysis in BSL-2 or -3 laboratories [6,17,25].

Results

### Musoke mVLPs induce strong homologous & heterologous humoral responses

Genetic differences in protective GP sequences are shown in FIGURE 1. Based on approximations from BLASTP pairwise comparisons of the translated GP genes, there is a 7% difference in identity between MARV-Musoke and -Ci67/Popp and a 22% difference in Musoke and Ravn.

To determine whether the mVLPs, based on the Musoke strain of MARV, could broadly protect against multiple strains of MARV, guinea pigs were vaccinated with mVLPs or iMARV-Musoke. As assessed by ELISA (FIGURE 2A–C), the mVLP- and iMARV-vaccinated guinea pigs had high levels of homologous MARV-Musoke and heterologous MARV-Ravn and -Ci67 antibodies (geometric mean titer for VLPs: 316,228, 31,622 and 100,000; or iMARV: 1,000,000, 100,000 and 100,000, respectively).

Musoke-based mVLPs & iMARV vaccines provided complete protection against lethal challenge with homologous & heterologous MARV challenges

Upon challenge with any of the three MARV viruses, the mVLP-vaccinated guinea pigs exhibited none of the classical symptoms of filovirus infection and were 100% protected from lethal MARV-Musoke, -Ci67 or -Ravn infection (FIGURE 2D–F). By contrast, guinea pigs vaccinated with RIBI adjuvant alone were highly susceptible to lethal MARV challenge and died within 6–12 days of infection (FIGURE 2D–F). When we evaluated the circulating viral titers 7 days after infection, the mVLP- and iMARV-vaccinated guinea pigs had no detectable virus in their plasma, unlike the guinea pigs vaccinated with RIBI adjuvant alone that obtained viremias of 10^6–10^7 pfu/ml (FIGURE 3A). Two random subjects out of each group were euthanized at day 6–7 after challenge for determination of pathology and organ viral titers. When we examined the viral titers of the tissues from guinea pigs that were vaccinated with mVLP or iMARV and RIBI adjuvant, we found a distinct lack of detectable virus in any of the tissues that we tested (FIGURE 3B–D). This was a consistent finding for both homologous MARV-Musoke (FIGURE 3B) or heterologous MARV-Ci67 or -Ravn challenge (FIGURE 3C & D). By stark contrast, the guinea pigs vaccinated with RIBI adjuvant only developed very high MARV titers in their liver, spleen, kidney, lymph nodes, testes, adrenal gland and lung, independent of the MARV isolate used to challenge. Only the guinea pigs challenged with MARV-Musoke had detectable virus in their pancreas (FIGURE 3B). Only one of two control guinea pigs infected with Ravn that was sampled had detectable virus in the brain tissue, while both of the MARV-Ci67 guinea pigs had detectable virus and neither of the MARV-Musoke guinea pigs had virus in their brain tissue (FIGURE 3B–D).

To confirm our findings that the mVLP- and iMARV-vaccinated guinea pigs were highly protected from MARV infection, we conducted macroscopic and microscopic
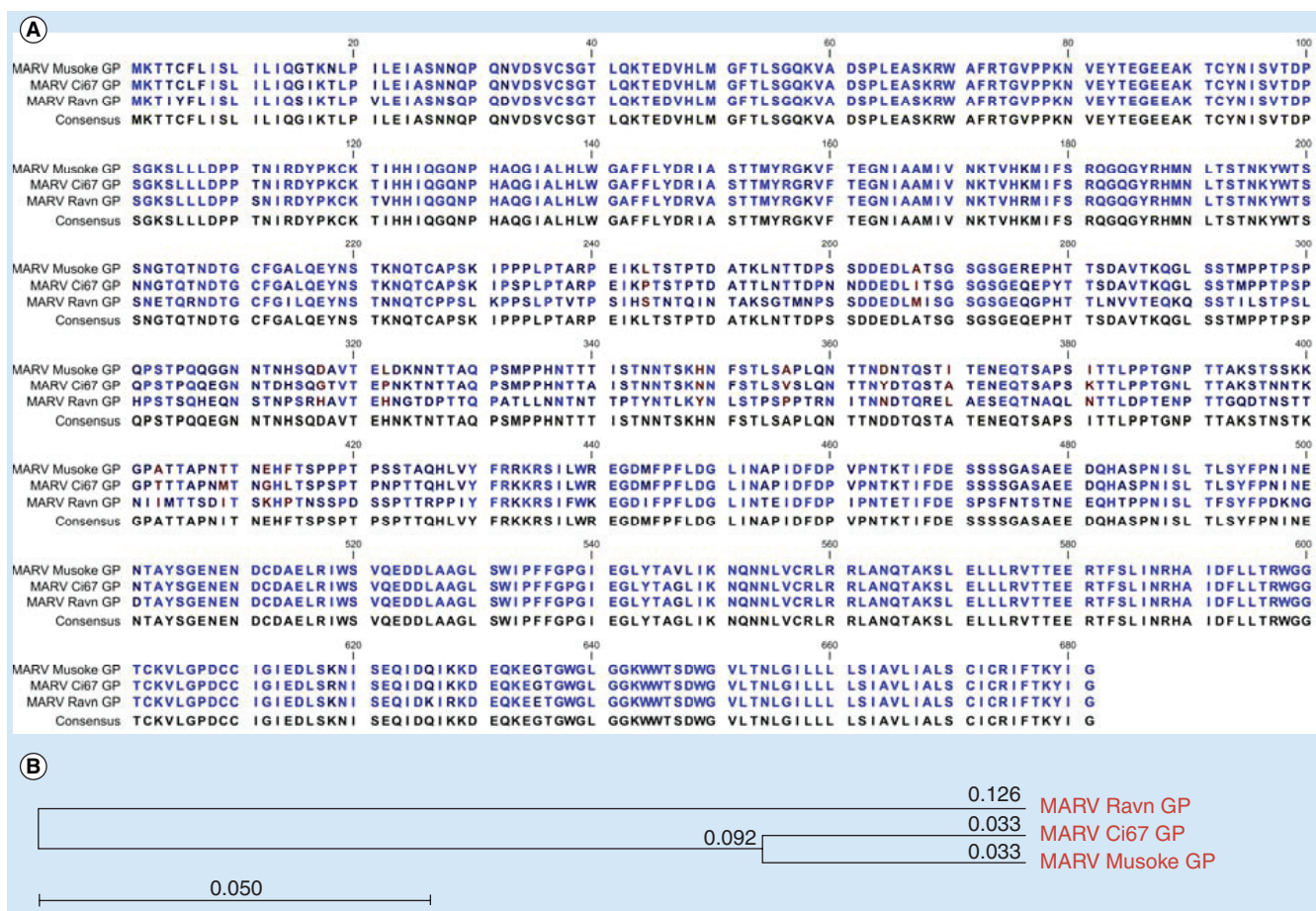
Figure 1. Sequence comparison of protective MARV GP molecules. (A) Alignment of predicted amino acid sequences for Musoke, Ci67 (Popp) and Ravn GPs. Identical sequences are shown in blue, conserved sequences in black and divergent sequences in red. The consensus sequence is shown at the bottom of the three aligned sequences. **(B)** Genetic tree showing the phlyogenetic relationship between the Musoke, Ci67 and Ravn GPs. Genetic distance is indicated on each branch.
MARV: Marburg virus; GP: Glycoprotein.

examinations of guinea pigs euthanized 6–7 days after challenge. The six guinea pigs administered RIBI adjuvant only and subsequently challenged with one of the three MARVs displayed typical, but nonspecific, signs of illness, including a rough hair coat, lethargy and weight loss. In addition, two animals displayed hind-limb paralysis and another two had mild ocular bleeding. Terminal bodyweights for the RIBI adjuvant-only treated and MARV-challenged animals were 150–300 g lower than for animals that were VLP-vaccinated and challenged with MARV (data not shown). A variety of macroscopic findings were noted at necropsy. The macroscopic findings were of low incidence and unrelated to the administration of RIBI, vaccine type or any of the three MARV isolates.

The protective effects of vaccination were most evident microscopically and immunohistochemically. Microscopic findings consistent with filovirus infection were noted in the liver, spleen, GI tract and in specific lymph nodes of RIBI adjuvant-only-vaccinated animals.

A mild-to-moderate hepatitis characterized by a mixed inflammatory response, hepatocellular degeneration and necrosis, and hepatocellular loss was multifocally scattered throughout the livers of the control animals challenged with all three virus isolates (FIGURE 4A). Rarely, hepatocytes contained eosinophilic cytoplasmic viral inclusions. Interestingly, the guinea pigs administered RIBI adjuvant only and challenged with MARV-Ravn had markedly blue cytoplasm in the hepatocytes peripheral to the foci of hepatocellular degeneration and necrosis (FIGURES 4A & B). The blue cytoplasm probably represents hepatocellular calcinosis. A mild-to-moderate vacuolar degeneration was present in all of the control animals and in three vaccinated animals; this change probably represents fat mobilization resulting from a diminished metabolic state (FIGURE 4A & B).

Mild-to-moderate lymphocytolysis was noted in the splenic white and red pulp, Peyer's patches and mesenteric lymph nodes of the RIBI control groups (FIGURE 4C). Some animals also had lymphoid depletion in these same tissues. In addition,
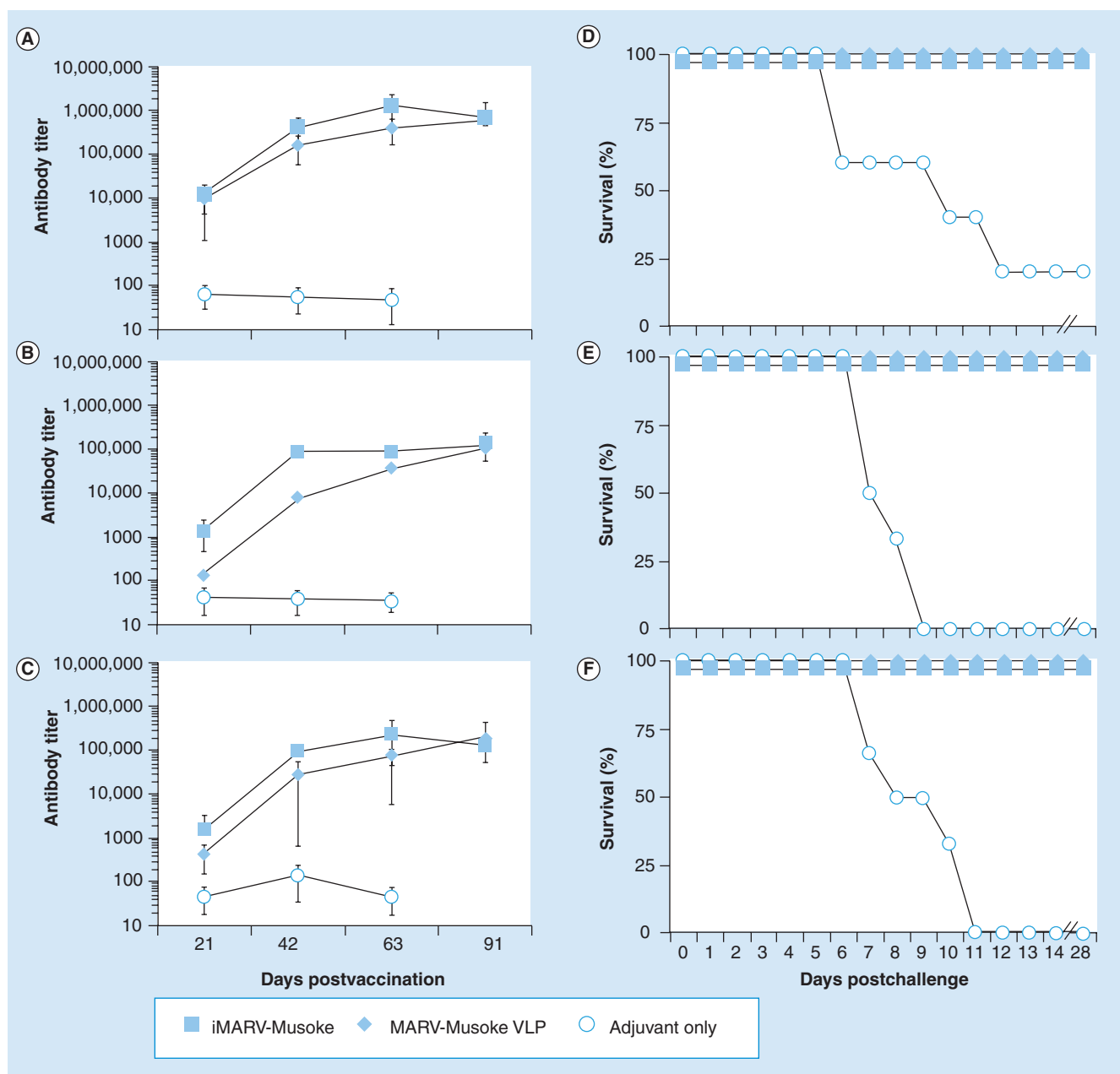
Figure 2. MARV-Musoke-based vaccines induce strong heterologous humoral responses in strain-13 guinea pigs. Animals were vaccinated with inactivated MARV-Musoke or MARV-Musoke VLPs in RIBI adjuvant (n = 27 each) or adjuvant only (n = 18), three-times at 3-week intervals. **(A–C)** Serum samples from the guinea pigs were obtained 3 weeks after the first (day 21), second (day 42) or third (day 63) vaccination and 4 weeks after challenge (day 91). Total serum **(A)** MARV-Musoke, **(B)** -Ravn or **(C)** -Ci67 antibodies were measured by ELISA. Antibody titers were measured in serum from individual guinea pigs and the results are graphed as the geometric mean end point titers. Error bars indicate standard deviation. **(D–F)** MARV-Musoke VLPs protect guinea pigs against multiple MARV isolates. The guinea pigs from each vaccine group were randomly divided into three challenge groups and challenged with 1000 pfu of guinea pig-adapted **(D)** MARV-Musoke, **(E)** -Ravn or **(F)** -Ci67, 4 weeks after the last vaccination. Results are presented on Meier–Kaplan curves as percentage survival over 28 days for each vaccination group (n = 6–7 per group).
iMARV: Inactivated Marburg virus; MARV: Marburg virus; pfu: Plaque-forming unit; VLP: Virus-like particle.

two animals in the control group had mild lymphocytolysis in the inguinal lymph node, and one of those had mild lymphoid depletion. Interestingly, the cervical lymph nodes were normal. In addition to the lymphocytolysis of the red and white pulp in the spleens, several unvaccinated animals demonstrated an increase in the number of neutrophils in the red

pulp. Four of the six RIBI adjuvant-only-vaccinated and MARV-challenged animals had mild cellular lysis of hematopoietic elements in the bone marrow. Five of the 12 vaccinated animals had a moderate increase in bone marrow neutrophils, interpreted to be an immunological response to the treatment and challenge protocol.

An uncommon finding of fibrin was present within the brain, spleen and mesenteric lymph node vasculature of five unvaccinated animals (TABLE 1). Two vaccinated and three unvaccinated animals had minimal-to-mild myocarditis. In contrast to the vaccinated animals, the foci of myocarditis in the unvaccinated (RIBI adjuvant-only) animals contained MARV antigen. However, the random incidence of the myocarditis among groups indicates that this is a background lesion that was populated with immunopositive macrophages in the unvaccinated groups.

A distinct difference between the vaccinees and the unvaccinated groups is evidenced by the lack of MARV-specific antigen in any of the animals in the vaccinated groups and a wide variety of antigen-positive tissue in the unvaccinated groups. Hepatocellular immunostaining was present on the cell surface and generally confined to hepatocytes surrounding foci of degeneration and necrosis (FIGURE 4B). MARV-specific antigen was commonly noted in macrophages and fibroblasts and specific cell types in a variety of tissues, as demonstrated in mesenteric lymph node and alveolar macrophages in FIGURE 4D & E. Immunopositive endothelial cells (FIGURE 4F) were present, but were not common. Inflammation and necrosis with associated immunopositive staining for MARV antigen was multifocally scattered throughout the gastrointestinal system (FIGURE 5A & B). Cells in the zona glomerulosa and zona fasciculata were immunopositive on their cell surfaces for MARV. The immunostaining of the adrenal gland was not associated with any morphological changes (FIGURE 5C & D). Epithelial cells in several tissues, including the esophagus, urinary bladder, skin and hair follicles, were immunopositive for MARV antigen (FIGURE 5E & F). The immunopositive epithelium was rarely associated with any inflammation or necrosis.
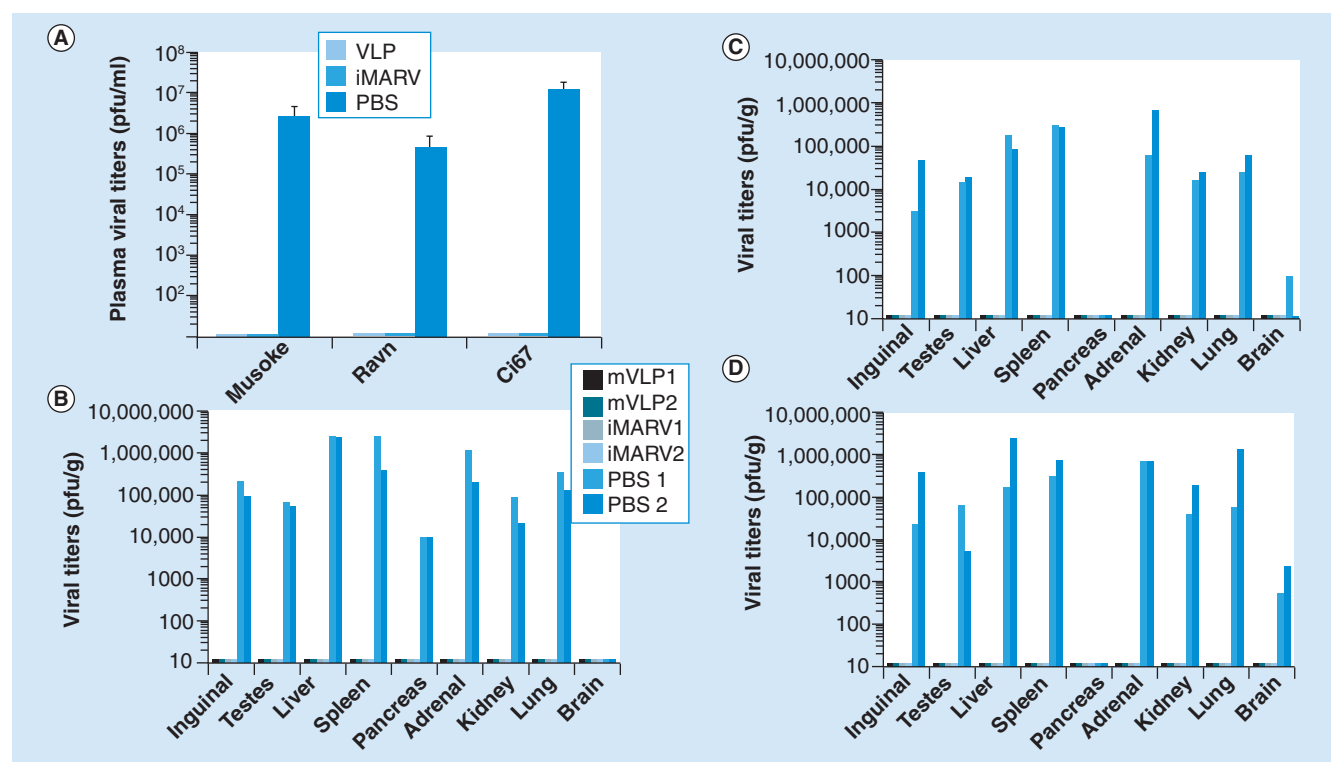
Figure 3. Lack of viral titers in mVLP- and iMARV-vaccinated guinea pigs at presumptive peak of viral replication. Viral titers in plasma or tissues from guinea pigs vaccinated with iMARV-Musoke or MARV-Musoke VLPs in RIBI adjuvant, or adjuvant only three-times at 3-week intervals. The guinea pigs from each vaccine group were randomly divided into three challenge groups and challenged with 1000 pfu of guinea pig-adapted **(B)** MARV-Musoke, **(C)** -Ravn, or **(D)** -Ci67, 4 weeks after the last vaccination. **(A)** Viremia was determined using standard plaque assay in the plasma of challenged guinea pigs at 7 days postchallenge (n = 6–7 per group). The data are presented as the mean viral titer with error bars representing the standard deviation. **(B–D)** Samples of the indicated tissues were taken upon necropsy at day 6 or 7 after infection of the mVLP, iMARV or PBS-RIBI adjuvant controls. The tissue samples were weighed, macerated and analyzed for viral titer using plaque assay. The data are presented as the viral titer in individual subjects sampled (n = 2 per group).
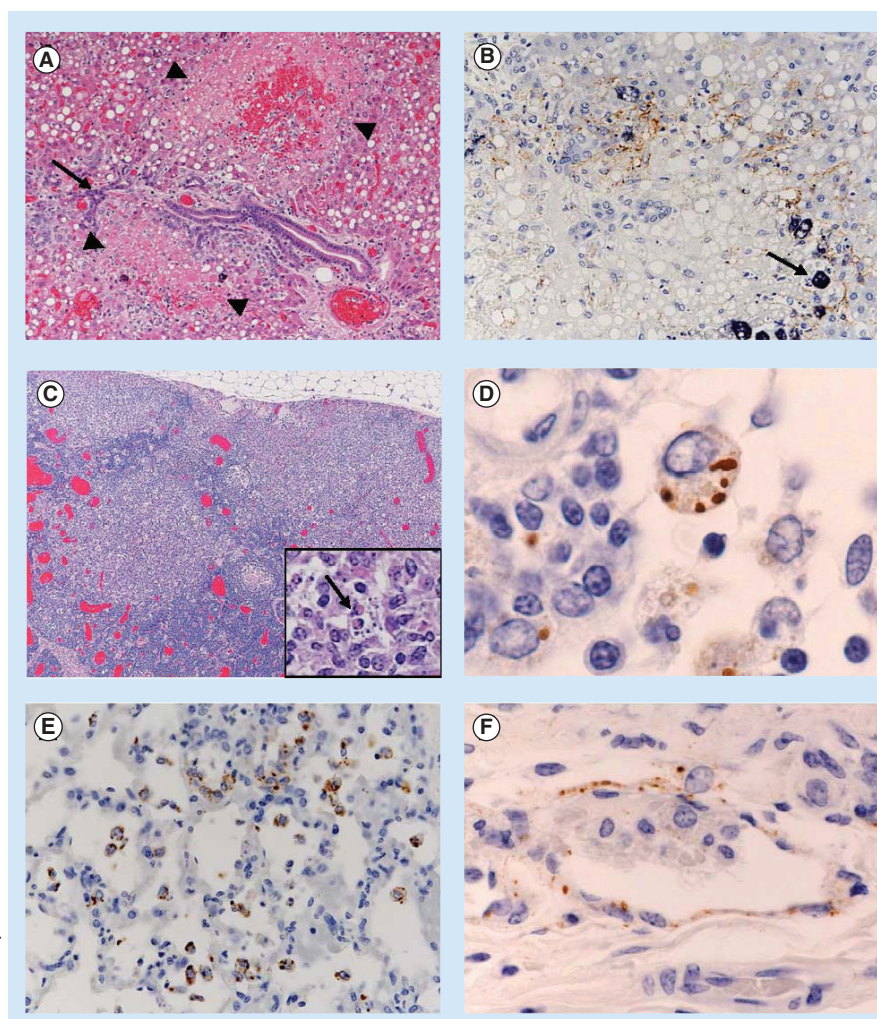iMARV: Inactivated Marburg virus; MARV: Marburg virus; mVLP: Marburg virus-like paricle; PBS: Phosphate-buffered saline; pfu: Plaque-forming unit; VLP: Virus-like particle.

Vaccination of NHPs with Musoke-based mVLPs & broad protection against multiple MARV isolates

Since the monovalent Musoke-based mVLPs provided such strong homologous and heterologous protection in guinea pigs, we wanted to determine whether mVLP vaccination could also provide broad protection against multiple MARV isolates. Following vaccination with mVLPs, antibody responses in mVLP-vaccinated macaques were determined using ELISA against irradiated MARV-Musoke, -Ci67 or -Ravn virions immediately before each vaccination and prior to challenge (TABLE 2 and data not shown). MARV-specific antibodies in the mVLP-vaccinated monkeys rose quickly after the first vaccination and homologous antibodies plateaued after two vaccinations (data not shown). Heterologous antibody titers rose and peaked after the third vaccination (TABLE 1 and data not shown). The nine mVLP-vaccinated monkeys were divided into three groups and challenged 4 weeks after the last vaccination with approximately 1000 pfu of MARV-Musoke, -Ci67 or -Ravn. The control monkey in each challenge group developed clinical and laboratory signs of MARV infection on days 8–10 after challenge (TABLE 2). The mVLP-vaccinated monkeys were protected from disease following lethal MARV challenge, except for a single monkey (410023). After challenge with MARV-Ravn, this monkey developed minor signs of disease, although no viremia was detected by plaque assay. By contrast, the controls in the experiment developed very high viral titers and also severe clinical, pathological and laboratory changes associated with MARV infection (TABLE 2 and data not shown).

Discussion

Previously, we showed that guinea pigs vaccinated with mVLPs or iMARV develop

Figure 4. Microscopic changes in control guinea pigs infected with Marbug virus (MARV). (A) Liver. Note the multifocal foci of hepatocellular degeneration and necrosis (outlined by arrowheads) with neutrophilic and monocytic inflammation and hemorrhage/congestion, and mild bile duct hyperplasia (arrows). Numerous hepatocytes contain large clear vacuoles (vacuolar degeneration). Hematoxylin and eosin staining, shown at 10×. **(B)** Liver. Hepatocytes stain immunohistochemically positive using antibodies against MARV and dark blue hepatocytes containing mineral deposition are peripheral to a focus of degeneration and necrosis (arrows). Immunoperoxidase method with hematoxylin counterstain, shown at 20×. **(C)** Mesenteric lymph node. The cortex has multifocal areas of lymphoid depletion and lymphocytolysis highlighted in the inset (arrows). Hematoxylin and eosin staining, shown at 2× with inset at 20×. **(D)** Mesenteric lymph node. Immunohistochemically positive macrophage within the medullary sinus. Immunoperoxidase method with hematoxylin counterstain, shown at 60×. **(E)** Lung. Immunohistochemically positive alveolar macrophages with mild interstitial thickening. Immunoperoxidase method with hematoxylin counterstain, shown at 20×. **(F)** Blood vessel. Tracheal submucosa showing immunohistochemically positive endothelial cells. Immunoperoxidase method with hematoxylin counterstain, shown at 20×.

homologous humoral and T-cell responses and are completely protected from a lethal homologous MARV challenge [18]. Protection from homologous challenge can even be afforded by a single injection of mVLPs in guinea pigs. The goal of the current work was to determine whether a VLP vaccine based on a single MARV isolate could be used to develop a vaccine that provides maximum and broad protection against diverse isolates of MARV. Here, we showed that Musoke isolate-based mVLPs could induce crossreactive antibodies and broadly protect guinea pigs and NHPs against multiple strains of MARV, including the MARV-Musoke, -Ravn and -Ci67 isolates. Not only did the Musoke-based mVLPs and iMARV vaccines provide complete protection in guinea pigs

Microscopic finding	mVLP + RIBI[*]			iMARV + RIBI[*]			RIBI[*]		
	Musoke[‡]	Ravn[‡]	Ci67[‡]	Musoke[‡]	Ravn[‡]	Ci67[‡]	Musoke[‡]	Ravn[‡]	Ci67[‡]
Brain									
Fibrin thrombi	-	-	-	-	-	-	-	-	++
Liver									
Hepatitis, mixed with degeneration and necrosis	-	-	-	-	-	-	+++/+++	+++/++	++/++
Viral inclusions (rare)	-	-	-	-	-	-	P/P	P/P	P/P
Vacuolar degeneration	++/-	+/+	-	-	-	+++/-	+++/+++	+++/+++	++/++
Hepatocellular calcinosis	-	-	-	-	-	-	-	P/P	-
Spleen									
Lymphocytolysis, white pulp	-	-	-	-	-	-	++/++	++/++	+++/+++
Lymphocytolysis, red pulp	-	-	-	-	-	-	+++/++	++/+++	+++/+++
Splenitis, neutrophilic	-	-	-	-	-	-	++/++	+++/-	+++/+++
Lymphoid depletion	-	-	-	-	-	-	-	-	++/++
Fibrin thrombi	-	-	-	-	-	-	-	-	++/++
Mesenteric lymph node									
Hemorrhage	-	-	-	-	-	-	-	-	+
Lymphoid depletion/lymphocytolysis	-	-	-	-	-	-	+++/+++	++/++	++/++
Fibrin thrombi	-	-	-	-	-	-	-	++/++	-
Peyer's patches									
Lymphoid depletion/lymphocytolysis	-	-	-	-	-	-	+++/+++	++/++	++/++
Bone marrow									
Hyperplasia, neutrophilic	-	+++/+++	-	-	+++/+++	+++/-	-	-	-
Cellular lysis	-	-	-	-	-	-	-	++/++	++/++
Stomach									
Cellular lysis	-	-	-	-	-	-	++/++	-/++	-/+
Gastritis, neutrophilic	-	-	-	-	-	-	+/++	+/++	-
Intestine									
Cellular lysis	-	-	-	-	-	-	+	+	+
Enteritis, neutrophilic	-	-	-	-	-	-	++/++	++/++	++/++
Heart									
Myocarditis	-	-/+	-	-	-/+	-	++/++	-/++	-

[*]Guinea pigs were vaccinated with three doses of the indicated immunogen in the presence of RIBI adjuvant or were administered RIBI adjuvant alone.
[‡]Guinea pigs were challenged with 1000 pfu of the indicated isolate of guinea pig-adapted MARV and two guinea pigs per group were euthanized on day 6–7 postchallenge for pathology studies.
Scoring system (noted for each guinea pig): -: negative/not noted; +: minimal; ++: mild; +++: moderate; ++++: marked; +++++: severe.
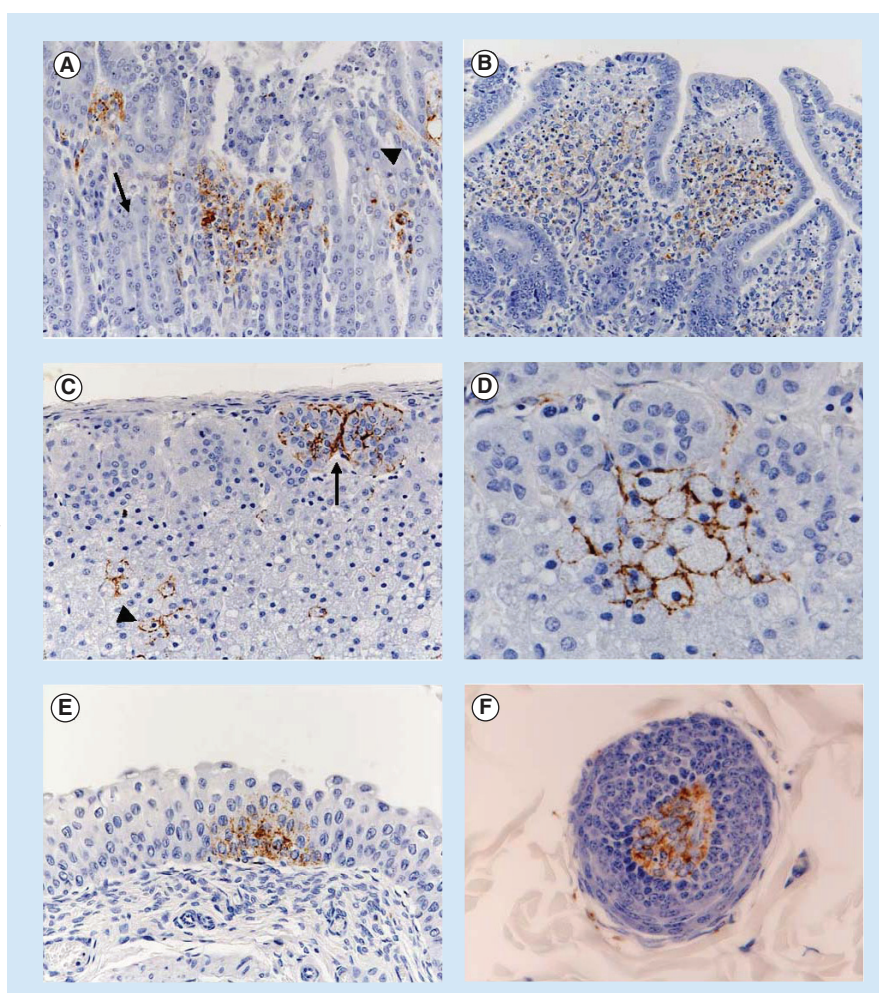LN: Lymph node; iMARV: Inactivated Marburg virus; MARV: Marburg virus; mVLP: Marburg virus-like particle; P: Present; pfu: Plaque-forming unit.

against overt clinical disease and death, but also from viremia, viral replication and pathological changes in tissues. Therefore, our Musoke MARV-based VLP vaccine was effective at inducing heterologous protective immune responses against multiple MARV isolates in both guinea pigs and NHPs.

Clinical, histological and immunohistological findings, restricted to six animals in the three RIBI-vaccinated control groups, were similar to those previously described in filovirus-infected guinea pigs [4,26–33]. The clinical findings were typical of what would be expected for filovirus-infected guinea pigs (except anorexia with weight loss, ruffled fur and lethargy) with the exception of the ocular bleeding in two of the 18 control animals and the hind-limb paralysis in at least two other control animals. The ocular bleeding was not present in any guinea pigs at necropsy and a source for the ocular bleeding noted clinically was not found, but could have been related to a retro-orbital bleed that occurred on day 7, nor was an etiology for the hind-limb paralysis evident in the evaluated brain tissue; however, a complete histopathologic evaluation of the central and peripheral nervous systems was not completed.

The six guinea pigs evaluated in this study had histopathologic findings similar to those seen in the NHP and mouse models for MARV infection (data not shown and [34–39]). Although morphologically similar to both the NHP (data not shown and [WARFIELD ET AL., UNPUBLISHED DATA; 34–38]) and mouse model [WARFIELD ET AL., UNPUBLISHED DATA; 39], hepatocellular degeneration and necrosis with accompanying inflammation was observed, although less frequently. In addition, the hepatocellular calcinosis was an interesting finding and specifically unique to those guinea pigs challenged with the MARV-Ravn isolate.

Figure 5. Microscopic changes in control guinea pigs infected with Marburg virus. **(A)** Stomach, glandular. Immunohistochemically positive gastric mucosa with inflammation and necrosis. Immunoperoxidase method with hematoxylin counterstain, shown at 20×. **(B)** Small intestine, villi. Immunohistochemically positive lamina propria with inflammation, necrosis and focal ulceration. Immunoperoxidase method with hematoxylin counterstain, shown at 20×. **(C)** Adrenal gland. Immunohistochemically positive cells within the zona glomerulosa (arrow) and zona fasciculata (triangle). Immunoperoxidase method with hematoxylin counterstain, shown at 20×. **(D)** Adrenal gland. Immunohistochemically positive cells demonstrating surface antigen on cells within the zona fasciculata (triangle). Immunoperoxidase method with hematoxylin counterstain, shown at 40×. **(E)** Urinary bladder. Immunohistochemically positive urothelium. Immunoperoxidase method with hematoxylin counterstain, shown at 20×. **(F)** Skin, hair follicle. Immunohistochemically positive dermal papilla within a hair follicle. Immunoperoxidase method with hematoxylin counterstain, shown at 40×.

As with the histologic lesions observed in the liver, lymphocytolysis and lymphoid depletion found in the spleen and some lymph nodes was similar to that found in both the NHP (data not shown and [34–38]) and mouse models [WARFIELD ET AL., UNPUBLISHED DATA; 39]. The immunostaining of epithelial cells in the skin, urinary bladder and esophagus is similar to that of the NHP model. However, the low presence of immunopositive hair follicles was a unique finding not previously observed in either the NHP or mouse model (data not shown). Fibrin deposition is a variable finding in filovirus infections, particularly in MARV-infected animals. Albeit less than observed in the NHP model, the histologic presence of fibrin in multiple tissues from these MARV-infected guinea pigs indicates some similarity to coagulation pathology in the monkey (data not shown and [34–38]). Although not performed in this current study, phosphotungstic acid hematoxylin stain for fibrin or identification of fibrin using immunohistochemical methods may better characterize the extent, or lack thereof, of fibrin deposition, not only in the brain, spleen and mesenteric lymph nodes, but in other tissues as well. Furthermore, and unlike

Table 2. Nonhuman primate study demonstrating broad protection against multiple Marburg virus isolates.

Monkey	Vaccine*	Challenge virus‡	End point ELISA antibody titers before challenge§			Time to death¶	Peak viremia# (pfu/ml)	Maximum ALT (fold change from day 0)**	Maximum ALP (fold change from day 0)**
			MARV-Musoke	MARV-Ci67	MARV-Ravn				
408239	mVLP (Musoke)	MARV-Musoke	5	4	4	NA	0	77 (2.08)	347 (1)
405271	mVLP (Musoke)	MARV-Musoke	4.5	4	4	NA	0	71 (2.09)	602 (1)
407032	mVLP (Musoke)	MARV-Musoke	5	4	4.5	NA	0	59 (1.84)	384 (1)
310340	mVLP (Musoke)	MARV-Ci67	4.5	4	4.5	NA	0	77 (1.6)	217 (1)
405278	mVLP (Musoke)	MARV-Ci67	4	3.5	4	NA	0	59 (1.51)	393 (1.03)
408030	mVLP (Musoke)	MARV-Ci67	5	4	4	NA	0	55 (2.11)	325 (1)
408531	mVLP (Musoke)	MARV-Ravn	5	4.5	4.5	NA	0	111 (2.78)	371 (1.09)
410023	mVLP (Musoke)	MARV-Ravn	4.5	4	4	NA	0	884 (21.04)	1136 (1.94)
409042	mVLP (Musoke)	MARV-Ravn	5	4.5	4.5	NA	0	86 (3.44)	444 (1)
407284	None	MARV-Musoke	1.5	2	1.5	10	1.2×10^9	886 (21.6)	1158 (3.59)
408199	None	MARV-Ci67	2.5	2	2	10	$>1 \times 10^{10}$	56 (1.3)	742 (2.26)
408497	None	MARV-Ravn	2	2	1.5	8	2.5×10^9	1073 (28.24)	1424 (3.72)

*Cynomolgus macaques were vaccinated with 1 mg of baculovirus-derived Marburg-Musoke VLPs containing GP, NP and VP40 in QS-21 adjuvant on days 0, 42 and 84.
‡Monkeys were challenged with approximately 1000 pfu of the indicated MARV isolate via subcutaneous injection on day 112.
§Circulating antibody titers against the indicated irradiated MARV antigen were determined by ELISA. Titers are expressed as the last $\log_{10}$ dilution where the optical density was greater than 0.200.
¶All vaccinated monkeys survived to day 28. Control monkeys 407284 and 408497 were euthanized and a terminal blood sample was obtained. Control monkey 408199 was found dead in the cage on day 10, so no terminal bleed was obtained (last bleed on day 7).
#Viremia was determined on days 0, 3, 5, 7, 10, 14 and 21 for all animals using standard plaque assay from serum samples.
**Liver functions were determined using chemistry 13 panels (Abaxis) on days 0, 3, 5, 7, 10, 14, 21 and 28. The maximum ALT and ALP levels for all timepoints are listed and compared with the day 0 value (expressed as fold change from day 0).
ALP: Alkaline phosphatase; ALT: Alanine aminotransferase; GP: Glycoprotein; MARV: Marburg virus; mVLP: Marburg virus-like particle; NA: Not applicable; NP: Nucleloprotein; VLP: Virus-like particle.

either the NHP or mouse model, gastrointestinal inflammation and necrosis was more commonly noted in the guinea pig and may represent an area of greater MARV susceptibility. More detailed clinical pathology and histomorphological evaluation of guinea pigs challenged with MARV will further their usefulness as an animal model for MARV infections.

Previously, we have demonstrated boosts in antibody titers following challenge and development of T-cell responses to additional viral proteins in VLP-vaccinated mice, although there was no evidence of viral infection at day 7 [40]. This indicated that, while the VLPs conferred protective immunity, the VLP vaccination did not provide sterilizing immunity in mice. However, in the current study, we showed that guinea pigs that were robustly vaccinated with mVLPs (three doses at 21-day intervals) did not develop viremia or detectable viral titers in their tissues following challenge. In addition, these guinea pigs did not develop increases in their postchallenge antibody titers, even when challenged with a heterologous virus (FIGURE 2A–C). Together, these data indicate vigorous homologous and heterologous immunity provided by VLP vaccination.

MARV-Musoke and EBOV-Zaire VLP vaccines provide homologous protection to NHPs (data not shown and [24]). We have also shown here that the Musoke-based mVLPs can broadly protect NHPs from at least three isolates of MARV (TABLE 2). Taken together with our current data and recent reports regarding homologous and heterologous protection by a Musoke-based vesicular stomatitis virus vaccine [7,8], we predict that Musoke-based mVLPs will provide broad protection to all known MARV isolates in NHPs and humans without substantial safety issues or toxicity. Utilization of a single Musoke-based mVLP vaccine will minimize the cost of vaccine production while maintaining effectiveness.

Expert commentary

Multiple vaccine approaches are efficacious in nonhuman primates against lethal EBOV and MARV infections. To date, the most successful filovirus vaccines have been based on viral vectors, such as adenovirus, venezulan equine encephalitis replicon, human parainfluenza type 3 and vesicular stomatitis virus.

To elicit protective immunity in nonhuman primates, and likely humans, there is a requirement for correct presentation of viral proteins, including the protective GP, as well as a sufficient vaccine dose. Successful vaccination regimens likely induce antibodies to assist in protection against the early phases of virus infection and also cytolytic T cells to destroy cells that do become infected with virus. Some of the many advantages of using VLPs as vaccines against filovirus infections include their similar morphology to the live viruses from which they are derived, a strong safety profile as they are nonreplicating, no viral vector or pre-existing antivector immunity concerns, the fact that they can be generated in large quantities using mammalian or insect cell lines, their generation of innate, humoral and cellular immunity, they have been safely and effectively administered to humans and have been approved by the FDA for use in humans, as in the case of the human papillomavirus (Gardasil® [101]) and hepatitis B virus. Our recent work has shown the utility of using the Ebola and Marburg VLPs as successful homologous vaccines in stringent NHP models. A monovalent mVLP vaccine is efficacious against challenge with multiple MARV isolates, including the most diverse MARV-Ravn. Thus, based on their safety, immunogenicity, and efficacy profiles, the filovirus VLPs are leading candidates for use as vaccines in humans.

Five-year view

We have shown here that the VLPs are highly immunogenic and can safely mediate protection of nonhuman primates from lethal EBOV and MARV infection, including broad protection against multiple and diverse MARV isolates. Based on immunogenicity and protective efficacy, as well as the known safety profile of VLPs in general from clinical trials, VLPs represent promise as the safest lead candidate filovirus vaccine for use in humans. The use of a nonreplicating subunit vaccine, such as the VLPs, may be highly advantageous not only for use in elderly, immunocompromised and young populations in the USA, but also for future use in Africa where the populations affected by filovirus outbreaks are often dehydrated, malnourished and immunocompromised due to concurrent parasitic and viral infections, including HIV. Work in NHPs will help

Key issues

- Filovirus virus-like particles (VLPs) for Ebola virus (EBOV)- and Marburg virus (MARV) containing combinations of proteins can be easily and efficiently made in mammalian or insect cells in large quantities.
- VLPs generate innate immune responses that can rapidly protect against filovirus infection.
- EBOV and MARV VLP vaccines activate virus-specific humoral and cell-mediated responses in rodents and nonhuman primates. Homologous (EBOV-Zaire) and heterologous (MARV-Musoke, -Ci67 and -Ravn) protection has been demonstrated for VLP vaccines in cynomolgus macaques.
- Since the efficacy of a filovirus vaccine cannot easily be evaluated in humans, studies to determine surrogate markers or correlates of protection in nonhuman primates will be required. These studies will help guide future biomarkers in clinical trials.
- So far, hepatitis B virus and human papillomavirus VLP vaccines are well-tolerated and effective in healthy, human volunteers and the safety profile of VLPs will permit their use in immunocompromised individuals.

guide correlates or surrogate markers of protection for future human clinical trials, where immunogenicity, but not efficacy, trials can occur. A clear profile regarding minimal levels of protective T-cell or antibody responses must be identified to determine the level and duration of protection conferred by the VLP vaccines. Future commercial development and clinical trials of the filovirus-like particle vaccines will be necessary to pave the way for use in humans.

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Disclaimer

Opinions, interpretations, conclusions and recommendations are those of the authors and are not necessarily endorsed by the US Army.

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

References

1   Slenczka W, Klenk HD. Forty years of Marburg virus. *J. Infect. Dis.* 196(Suppl. 2), S131–S135 (2007).

2   Hevey M, Negley D, Vanderzanden L *et al.* Marburg virus vaccines, comparing classical and new approaches. *Vaccine* 20, 586–593 (2002).

3   Ignat'ev GM, Strel'tsova MA, Agafonov AP, Zhukova NA, Kashentseva EA, Vorob'eva MS. [The immunity indices of animals immunized with the inactivated Marburg virus after infection with homologous virus]. *Vopr. Virusol.* 39(1), 13–17 (1994).

4   Ignatev GM, Agafonov AP, Strel'tsova MA *et al.* A comparative study of the immunological indices in guinea pigs administered an inactivated Marburg virus. *Vopr. Virusol.* 36, 421–423 (1991).

5   Ignatyev GM, Agafonov AP, Streltsova MA, Kashentseva EA. Inactivated Marburg virus elicits a nonprotective immune response in rhesus monkeys. *J. Biotechnol.* 44(1–3), 111–118 (1996).

6   Hevey M, Negley D, Pushko P, Smith J, Schmaljohn A. Marburg virus vaccines based upon alphavirus replicons protect guinea pigs and nonhuman primates. *Virology* 251(1), 28–37 (1998).

7   Jones SM, Feldmann H, Stroher U *et al.* Live attenuated recombinant vaccine protects nonhuman primates against Ebola and Marburg viruses. *Nat. Med.* 11(7), 786–790 (2005).

8   Daddario-DiCaprio KM, Geisbert TW, Geisbert JB *et al.* Cross-protection against Marburg virus strains by using a live, attenuated recombinant vaccine. *J. Virol.* 80(19), 9659–9666 (2006).

9   Wang D, Hevey M, Juompan LY *et al.* Complex adenovirus-vectored vaccine protects guinea pigs from three strains of Marburg virus challenges. *Virology* 353(2), 324–332 (2006).

10   Swenson DL, Wang D, Luo M *et al.* Complete protection of nonhuman primates against multi-strain Ebola and Marburg virus infections. *Clin. Vaccine Immunol.* 15(3), 460–467 (2008).

11   Hart MK. Vaccine research efforts for filoviruses. *Int. J. Parasitol.* 33, 583–595 (2003).

12   Warfield KL, Swenson DL, Demmin G, Bavari S. Filovirus-like particles as vaccines and discovery tools. *Expert Rev. Vaccines* 4(3), 429–440 (2005).

13   Hevey M, Negley D, Geisbert J, Jahrling P, Schmaljohn A. Antigenicity and vaccine potential of Marburg virus glycoprotein expressed by baculovirus recombinants. *Virology* 239(1), 206–216 (1997).

14   Riemenschneider J, Garrison A, Geisbert J *et al.* Comparison of individual and combination DNA vaccines for *B. anthracis*, Ebola virus, Marburg virus and Venezuelan equine encephalitis virus. *Vaccine* 21(25–26), 4071–4080 (2003).

15   Swenson DL, Warfield KL, Kuehl K *et al.* Generation of Marburg virus-like particles by co-expression of glycoprotein and matrix protein. *FEMS Immunol. Med. Microbiol.* 40(1), 27–31 (2004).

16   Swenson DL, Warfield KL, Negley DL, Schmaljohn A, Aman MJ, Bavari S. Virus-like particles exhibit potential as a pan-filovirus vaccine for both Ebola and Marburg viral infections. *Vaccine* 23(23), 3033–3042 (2005).

17   Bavari S, Bosio CM, Wiegand E *et al.* Lipid raft microdomains: a gateway for compartmentalized trafficking of Ebola and Marburg viruses. *J. Exp. Med.* 195(5), 593–602 (2002).

18   Warfield KL, Swenson DL, Negley DL, Schmaljohn AL, Aman MJ, Bavari S. Marburg virus-like particles protect guinea pigs from lethal Marburg virus infection. *Vaccine* 22(25–26), 3495–3502 (2004).

19   Moe JB, Lambert RD, Lupton HW. Plaque assay for Ebola virus. *J. Clin. Microbiol.* 13(4), 791–793 (1981).

20   Lofts LL, Ibrahim MS, Negley DL, Hevey MC, Schmaljohn AL. Genomic differences between guinea pig lethal and nonlethal Marburg virus variants. *J. Infect. Dis.* 196(Suppl. 2), S305–S312 (2007).

21   Warfield KL, Posten NA, Swenson DL *et al.* Filovirus-like particles produced in insect cells: immunogenicity and protection in rodents. *J. Infect. Dis.* 196(Suppl. 2), S421–S429 (2007).

22 National Research Council. *Guide for the Care and Use of Laboratory Animals* (1996).

23 Warfield KL, Swenson DL, Olinger GG *et al.* Gene-specific countermeasures against Ebola virus based on antisense phosphorodiamidate morpholino oligomers. *PLoS Pathog.* 2(1), e1 (2006).

24 Warfield KL, Swenson DL, Olinger GG, Kalina WV, Aman MJ, Bavari S. Ebola virus-like particle-based vaccine protects nonhuman primates against lethal Ebola virus challenge. *J. Infect. Dis.* 196(Suppl. 2), S430–S437 (2007).

25 Rao M, Bray M, Alving CR, Jahrling P, Matyas GR. Induction of immune responses in mice and monkeys to Ebola virus after immunization with liposome-encapsulated irradiated Ebola virus: protection in mice requires CD4+ T cells. *J. Virol.* 76(18), 9176–9185 (2002).

26 Simpson DI, Zlotnik I, Rutter DA. Vervet monkey disease: experimental infection of guinea pigs and monkeys with the causative agents. *Br. J. Exp. Pathol.* 49, 458–464 (1968).

27 Zlotnik I. Marburg agent disease: pathology. *Trans. R. Soc. Trop. Med. Hyg.* 63, 310–323 (1969).

28 Robin Y, Bres P, Camain R. Passage of Marburg virus in guinea pigs. In: *Marburg Virus.* Martini GA, Siegert R (Eds). Springer-Verlag, NY, USA 117–122 (1971).

29 Connolly BM, Steele KE, Davis KJ *et al.* Pathogenesis of experimental Ebola virus infection in guinea pigs. *J. Infect. Dis.* 179(Suppl. 1), S203–S217 (1999).

30 Volchkov VE, Chepurnov AA, Volchkova VA, Ternovoj VA, Klenk HD. Molecular characterization of guinea pig-adapted variants of Ebola virus. *Virology* 277(1), 147–155 (2000).

31 Ryabchikova E, Kolesnikova L, Smolina M *et al.* Ebola virus infection in guinea pigs: presumable role of granulomatous inflammation in pathogenesis. *Arch. Virol.* 141(5), 909–921 (1996).

32 Ryabchikova E, Strelets L, Kolesnikova L, P'yankov O, Sergeev A. Respiratory Marburg virus infection in guinea pigs. *Arch. Virol.* 141(11), 2177–2190 (1996).

33 Slenczka W, Shu HL, Piepenberg G, Siegert R. Detection of the antigen of the "Marburg virus" in the organs of infected guinea-pigs by immunofluorescence. *Ger. Med. Mon.* 13(11), 524–529 (1968).

34 Gedigk P, Bechtelsheimer H, Korb G. [Pathological anatomy of the "Marburg virus" disease (the so-called "Marburg monkey disease")]. *Dtsch. Med. Wochenschr.* 93(12), 590–601 (1968).

35 Shu HL, Siegert R, Slenczka W. The pathogenesis and epidemiology of the "Marburg-virus" infection. *Ger. Med. Mon.* 14(1), 7–10 (1969).

36 Haas R, Maass G. Experimental infection of monkeys with the Marburg virus. In: *Marburg Virus.* Martini GA, Siegert R (Eds). Springer-Verlag, NY, USA 136–143 (1971).

37 Murphy FA, Simpson DI, Whitfield SG, Zlotnik I, Carter GB. Marburg virus infection in monkeys. Ultrastructural studies. *Lab. Invest.* 24(4), 279–291 (1971).

38 Stille W, Bohle E. Clinical course and prognosis of Marburg virus ("green-monkey") disease. In: *Marburg Virus Disease.* Martini GA, Siegart R (Eds). Springer-Verlag, NY, USA 10–18 (1971).

39 Warfield KL, Alves DA, Bradfute SB *et al.* Development of a model for marburgvirus based on severe-combined immunodeficiency mice. *Virol. J.* 4(1), 108 (2007).

40 Warfield KL, Olinger G, Deal EM *et al.* Induction of humoral and CD8+ T cell responses are required for protection against lethal Ebola virus infection. *J. Immunol.* 175(2), 1184–1191 (2005).

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